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[Note]

L-Phenylalanine and L-tyrosine degradative pathways in *Rhodococcus erythropolis*

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A Gram-positive *Rhodococcus erythropolis* strain S1 was shown to degrade L-phenylalanine via phenylpyruvate and homogentisate, and L-tyrosine through *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and homogentisate, which were conducted from oxygen consumption with intact cells, thin layer chromatography, and high performance liquid chromatography experiments. These pathways are in good agreement with that observed in other Gram-positive bacteria such as *Nocardia* sp. and *Streptomyces* sp.. L-Phenylalanine and L-tyrosine would appear to be transaminated by aromatic amino acid oxidase. Aromatic amino acid oxidase, *p*-hydroxyphenylacetate 1-hydroxylase, and homogentisate 1,2-dioxygenase activities were inducible by L-tyrosine or L-phenylalanine in strain S1.

In recent years, many microorganisms have appeared to play an important role as a degrader of aromatic compounds in the environment, while there has been little available information on the degradation of aromatic compounds by Gram-positive bacteria and actinomycetes. On the other hand, catabolism of aromatic amino acids, phenylalanine and tyrosine have been studied extensively in mammals, plants, and Gram-negative bacteria[1,2].

Until now, Gram-positive *R. erythropolis* S1 was isolated from soil using the enrichment procedure with phthalate ester as the sole carbon source[3], and has been shown to be capable of assimilating not only various aromatic carboxylic acids such as monohydroxybenzoates[4], dihydroxybenzoastes[5,6], and phthalate[7], but also aromatic amino acids, phenylalanine, tyrosine, and tryptophan[8]. This paper describes the identification of the degradative pathways of L-phenylalanine and L-tyrosine in strain S1.

YMG-medium was composed of 4 g yeast extract (Difco), 10 g malt extract (Difco), and 4 g glucose in 1 liter of distilled water, pH 7.3. Minimal medium composition was described previously[9]. L-Phenylalanine and L-tyrosine were purchased from Wako Pure Chemical Company (Japan). Phenylpyruvate, *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetic acid, homogentisate, and homoprotocatechuate were obtained from Sigma (U.S.A.). The strain S1 was preincubated in YMG-medium with shaking at 30°C for 1 day. One ml of the YMG-preculture was inoculated in 100 ml of minimal medium with 0.1 % L-tyrosine, and incubated with shaking at 30°C for 16 hr. On the other hand, cells in the YMG-culture of 2400 ml were harvested by centrifugation (5000*g* for 10 min at 10°C), washed twice with distilled water, and suspended at a concentration of 1 % of wet-cells per ml in minimal medium containing 0.3 % L-phenylalanine. The suspension was incubated with shaking at 30°C for 2-3 days. Growth of cells was monitored by measuring the optical density with the absorbance at 660 nm (O.D.660). Oxygen uptake experimetns was carried out using cells grown on L-phenylalanine, L-tyrosine, or intermediates. Cells in the logarithmic period were harvested in centrifugation and washed with distilled water twice. Washed cells were rapidly used for oxygen uptake experiment. The assay system for oxygen uptake by intact cells grown on L-phenylalanine, L-tyrosine, or various intermediates contained a suitable amount of washed cells and 2 mM substrate in a final volume of 2 ml of 10 mM sodium phosphate buffer, pH 7.1. The oxygen consumption was monitored with a biological oxygen monitor (YSI model 5300). As shown in Table 1, Cells grown on L-phenylalanine rapidly consumed oxygen in the presence of L-phenylalanine, phenylpyruvate, and homogentisate. Otherwise, *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and homoprotocatechuate were not almost utilized by L-phenylalanine-grown cells. Phenylpyruvate-grown cells appeared to be well adapted with L-phenylalanine and homogentisate as well as the L-phenylalanine-grown cells, but homogentisate-grown cells could not utilize L-phenylalanine or phenylpyruvate. Cells grown on L-tyrosine showed the full consumption of oxygen with L-tyrosine, *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and homogentisate, while L-phenylalanine, phenylpyruvate, and homoprotocatechuate were not utilized by L-tyrosine-grown cells. Cells grown on *p*-hydroxypheylpyruvate were adapted for the utilization of L-tyrosine, but *p*-hydroxyphenylacetate- or homogentisate-grown cell were not able to adapted for the utilization of L-tyrosine. The results are in good agreement with

previous reports [10], and suggested that the catabolism of L-phenylalanine and L-tyrosine occured by a pathway involving homogentisate as a key intermediate.

Aromatic amino acids and related intermediates in L-phenylalanine- or L-tyrosine-culture were determined by using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analysis methods. Culture broth was acidified to pH 1 to 2, extracted twice with ether, and concentrated by evaporation. Samples were applied to TLC plates. TLC was performed on silica gel plates of TLC RP8 254S (Merck) with a mobile phase of acetonitorile/water (6:4) or of HPTLC NH2 254S (Merck) with a mobile phase of ethanol/ammonia (6:4) containing 0.18 M NaCl. Aromatic compounds were detected by UV absorption at 254 nm. HPLC was performed on a microbore reverse phase column (ODS 80TM, Tosoh) with a linear 10-50 % (v/v) gradient in 50 mM KH2PO4 for 40 min at 0.5 ml per min. Wavelengths for the chromatograms were 274 nm for L-tyrosine and 253 nm for L-phenylalanine intermediates [11]. Intermediates presented in L-phenylalanine-culture were conducted to be phenylpyruvate and homogentisate. Also, L-tyrosine was determined to be converted to *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and homogentisate. These results were good in agreement with the oxygen uptake experiments by using the intact cells grown on L-phenylalanine or L-tyrosine. Therefore, it was concluded that L-phenylalanine was degraded via phenylpyruvate and homogentisate, and also that L-tyrosine was degraded through *p*-hydroxyphenylpyruvate, *p*-hydroxyphenylacetate, and homogentisate by strain S1 (Fig. 1). These L-phenylalanine and L-tyrosine degradative pathways have been published not only from Gram-negative bacteria but also from *Streptomyces* sp. We could not determine whether phenylpyruvate converted from L-phenylalanine was degraded via phenylacetate because we could not obtain phenylacetic acid as commercial products. In addition, phenylacetate was not appeared to be hydroxylated to homogentisate by one step or two steps of hydroxylation [12,13].

Crude extracts from the cells grown on L-phenylalanine or L-tyrosine was prepared by the method described previously [14]. Aromatic amino acid oxidase activity was assayed spectrophotometrically with the method of Brearly *et al.* [15], which was based on the measurement of H_2O_2. Phenylalanine dehydrogenase activity was measured spectrophotometrically by the method of Asano *et al.* [16], which assayed the oxidative deamination by reduction of NAD^+. *p*-Hydroxyphenylacetic acid 1-hydroxylase and homogentisate 1,2-dioxygenase activities were monitored by the same procedure described previously [4,6].

Crude extracts prepared from the L-phenylalanine- or L-tyrosine-grown cells was measured about various enzyme activities (Table 2). Both L-phenylalanine- and L-tyrosine-grown cells appeared to have aromatic amino acid (L-phenylalanine or L-tyrosine) oxidase activity, while not aromatic amino acid dehydrogenase or aminotransferase activities. It was not concluded that L-phenylalanine and L-tyrosine were oxidized by the identical enzyme, aromatic amino acid oxidase which has exhibited the broad substrate specificity [17]. The crude extracts from L-phenylalanine- or L-tyrosine-grown cells also indicated the *p*-hydroxyphenylacetate 1-hydroxylase activity [18]. But it was not concluded that *p*-hydroxyphenylacetate was intermediate in the L-phenylalanine degradative pathways because *p*-hydroxyphenylacetate was not detected in the L-phenylalanine-culture. *p*-Hydroxyphenylacetate 1-hydroxylase activity was observed only with the presence of NADH, therefore, this enzyme

Table 1. Oxygen uptake by intact cells grown on L-phenylalanine, L-tyrosine, intermediates, and other aromatic compounds.

Substrate	Specific oxygen uptake (nmol/min/ml/O.D.660)					
	L-Phenylalanine-grown	Phenylpyruvate-grown	L-Tyrosine-grown	*p*-Hydroxyphenyl pyruvate-grown	*p*-Hydroxyphenyl acetate-grown	Homogentisate-grown
L-Phenylalanine	68	54	6	6	4	2
Phenylpyruvate	63	78	9	5	6	3
L-Tyrosine	2	3	76	63	15	5
p-Hydroxyphenyl pyruvate	5	3	71	80	21	8
p-Hydroxyphenyl acetate	45	42	87	90	96	16
Homogentisate	106	114	165	148	184	209
Homoprotocatechuate	8	5	6	9	3	12
trans-Cinnamate	2	1	2	2	1	3

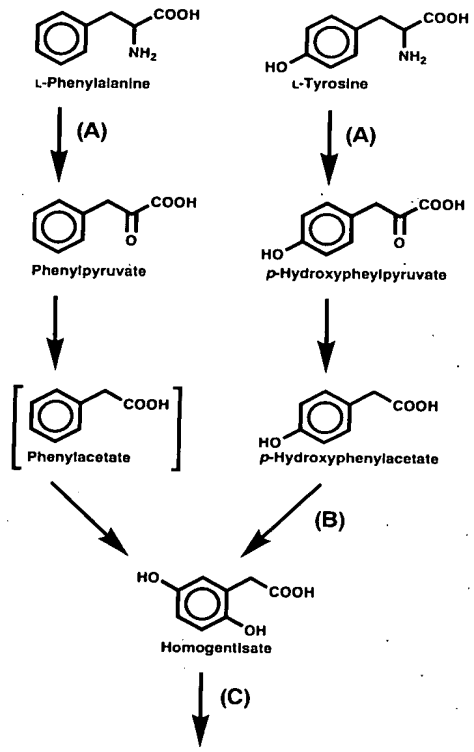

Fig. 1 L-Phenylalanine and L-tyrosine degradative pathways in *R. erythropolis* S1 (A) Aromatic amino acid oxidase; (B) *p*-Hydroxyphenylacetic acid 1-hydroxylase; (C) Homogentisate 1,2-dioxygenase.

in the strain S1 may be require strictly NADH. Homogentisate 1,2-dioxygenase activity was also detected in all crude extracts [19]. However, aromatic amino acid oxidase, *p*-hydroxyphenylacetate 1-hydroxylase, and homogentisate 1,2-dioxgenase activities were not detected in the crude extracts prepared from the cells grown in YMG-medium. These enzymes would therefore appear to be inducible by L-phenylalanine or L-tyrosine.

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Table 2. Various enzyme activities in crude extracts prepared from L-phenylalanine-, L-tyrosine-, and YMG-medium-grown cells of *R. erythropolis* S1

Enzyme	Activity (U/ml)		
	L-Phenylalanine-grown	L-Tyrosine-grown	YMG-medium-grown
Aromatic amino acid oxidase	89[a]	65[a]	9
	69[b]	74[b]	8
L-Phenylalanine dehydrogenase	2	1	< 1
p-Hydroxyphenylacetic acid 1- hydroxylase	143	106	4
Homogentisate 1,2-dioxygenase	261	297	6

a: activity was measured by using L-phenylalanine as substrate.

b: activity was measured by using L-tyrosine as substrate.

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